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Title: **p90 RSK INHIBITORS AS THERAPEUTICS AND INVESTIGATIVE TOOLS**

25 Sheets of specification.
12 Sheets of drawings.

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p90 RSK Inhibitors as Therapeutics and Investigative Tools

Background

Improper regulation of the Mitogen-activated Protein Kinase (MAPK) pathway is a distinguishing characteristic in many tumors as well as neurological diseased states such as epilepsy. p90 Ribosomal S6 Kinase (RSK) is a downstream component of the MAPK signaling pathway and is activated by MAPK. Therefore, unregulated stimulation of the MAPK pathway results in unregulated RSK catalytic activity. The contribution of upstream components such as Epidermal Growth Factor Receptor (EGFR) and the products of the proto-oncogenes c-src, ras, and raf to diseased states has been well documented. Activation of any of these signaling molecules results in physiological responses by the cell. However, the extent to which these physiological responses function through RSK is unknown.

The paucity of data concerning key biological roles of the Ser/Thr protein kinase Rsk family in somatic cells results primarily from the difficulty in distinguishing Rsk function from those of MAPK itself and of the many other downstream MAPK effectors. This difficulty has arisen because of the lack of any Rsk-specific inhibitors. Accordingly, a RSK specific inhibitor is highly desirable for use as a tool for investigating RSK function under normal conditions and under diseased conditions in which regulation of the MAPK signaling pathway has been compromised. The present invention provides a method for screening for such inhibitors and identifies Rsk-specific inhibitors and the use of composition comprising such inhibitors for the treatment of diseases associated with elevated RSK activity.

Brief Summary of the Invention

A methanolic extract from the plant *Forsteronia refracta* has been discovered to contain several potent RSK inhibitors. The compounds have been shown to be specific inhibitors of RSK *in vitro* and *in situ*. In addition, inhibition of RSK by the compounds halts proliferation of cancer cell lines while having little effect on the proliferation rate of normal cells. Therefore, the present invention identifies RSK as a target for therapeutic intervention in diseased states in which the disease or the symptoms can be ameliorated by inhibition of RSK catalytic activity or

in combination with additional therapies. Any compound for which direct inhibition of RSK constitutes all or part of its physiological effect can fulfill this objective. This includes, but is not limited to the compounds or analogs thereof from the plant extract described herein.

Brief Summary of the Drawings

Fig. 1: Molecular structure of SL0101-1, SL0101-2 and SL0101-3

Fig. 2: Inhibitory potency of SL0101-1, SL0101-2 and SL0101-3. The catalytic activity of RSK in the presence of increasing concentrations of each compound was measured. The IC_{50}s of each compound was determined.

Fig. 3: *In vitro* specificity of SL0101. The influence of SL0101 on the catalytic activity of several protein kinases was examined.

Fig. 4: In situ examination of SL0101 efficacy. SL0101 reduced phosphorylation of the in vivo RSK substrates Estrogen Receptor a (ER a) and pp140. Phosphorylation of ER a and pp140 was detected with anti-phospho-specific antibody recognizing the RSK phosphorylation site in these substrates. Presence of the inhibitor did not alter phosphorylation of RSK as indicated by the reduced mobility of RSK during SDS-PAGE. SL0101 did not inhibit phosphorylation of MAPK as indicated by the anti-phospho-specific antibody recognizing phosphorylated, active MAPK.

Fig. 5: SL0101 inhibits proliferation of transformed cells but not parental cells. Inhibition of RSK by SL0101 halts proliferation of Ha-ras-transformed NIH/3T3 cells but has little effect on the proliferation rate of non-transformed NIH/3T3 cells compared to that observed with vehicle alone. Proliferation was measured using Promega CellTiter-Glo™ Luminescent cell viability assay.

Fig. 6. Identification and characterization of SL0101-1. a, Kinase assays were performed using immobilized substrate. The extent of phosphorylation was determined using phosphospecific antibodies directly labelled with horseradish peroxidase (HRP)-conjugated or phosphospecific antibodies in combination with HRP-conjugated secondary antibodies. All assays measured the initial reaction velocity. b, Assays were performed as described in a. Maximum activity was measured in the presence of vehicle. c, Assays were performed as

described in b in the presence of vehicle or extract and varying concentrations of ATP. d, The potency of the purified compounds and kaempferol to inhibit Rsk catalytic activity was measured in assays as described in b. e, Structure of SL0101-1. f, Kinase assays were performed as described in b in the presence of vehicle, 2 M SL0101-1 or 10 M H89.

Fig. 7. SL0101-1 inhibits activity of the amino-terminal kinase domain. a, HA-tagged Rsk2 and an HA-tagged truncation mutant containing the NTKD (Rsk2 (1-389)) were transfected into baby hamster kidney 21 (BHK21) cells. The HA-tagged proteins were immunoprecipitated from lysates of EGF-stimulated cells. Assays were performed as described in Fig. 6b in the presence of vehicle, 2 M SL0101-1 or 2 M Ro 318220. b, The adenosine interacting loop of Rsk is modeled from the crystal structure of PKA. c, HA-tagged proteins were immunoprecipitated from the lysates of EGF-stimulated BHK21 cells transiently transfected with the indicated HA-tagged constructs. Assays were performed as described in Fig. 6b in the presence of vehicle, 2 M SL0101-1 or 2 M Ro 318220.

Fig. 8. SL0101-1 preferentially inhibits cancer cell proliferation. a, MCF-7 and MCF-10A cells were pre-incubated with vehicle, 50 M U0126 or the indicated concentration of SL0101-1 for 3 hr. Cells were treated with 500 nM PDB for 30 min prior to lysis. Protein concentration of lysates was measured and lysates were electrophoresed, transferred and immunoblotted. Equal loading of lysate is demonstrated by the anti-Ran immunoblot. b, Ha-Ras-transformed NIH/3T3 cells and parental NIH/3T3 cells were treated with 50 M SL0101-1 or vehicle and cell viability measured at the indicated time points. c, Ha-Ras-transformed NIH/3T3 cells were treated with vehicle, 50 M SL0101-1 or 50 M U0126. Images were taken 48 hr after treatment at 20X magnification. d, Ha-Ras-transformed and NIH/3T3 cells were treated with vehicle or 50 M SL0101-1. After 48 hr the medium was replaced and cells previously incubated with vehicle were maintained in vehicle. Cells that had previously been incubated with SL0101-1 were treated with either SL0101-1or vehicle (washout). Cell viability was measured 48 hr later. e, MCF-7 and MCF-10A cells were treated with vehicle or 100 M SL0101-1 and cell viability measured at indicated time points. f, Duplex siRNAs to a sequence in the bluescript plasmid (Control), Rsk1 and Rsk2 were transfected into MCF-7 cells. Medium was replaced 24 hr post-transfection and the cells incubated for an additional 48 hr prior to measuring cell viability.

Fig. 9 Rsk is a potential drug target for breast cancer. a, MCF-7 and MCF-10A cells were treated with vehicle or the indicated compounds. Cell viability was measured 72 hr after treatment. Proliferation is represented as a percentage of that in the presence of vehicle. b, Normal (N) and cancerous breast tissue (LC, lobular carcinoma; MC, mucinous carcinoma; A, adenocarcinoma) was ground under liquid nitrogen and lysed in SDS-loading buffer. Protein concentration of lysates was measured and lysates were electrophoresed, transferred and immunoblotted. Equal loading of lysate is demonstrated by the anti-Ran immunoblot. c, MCF-7 cells were treated with vehicle or 50 M SL0101-1 in serum-free medium. Cell viability was measured at indicated time points.

Fig. 10 SL0101completely inhibits the proliferation of LNCaP cells but not C4-2 cells. LNCaP and C4-2 cells were treated with vehicle or 100 M SL0101 in the presence of 1 nM of the synthetic androgen, R1881. A proliferation assay using CellTiter-Glo Luminescent Cell Viability Assay (Promega) was performed and the data expressed relative to time 0.

Fig. 11 The RSK signal transduction pathway is dis-regulated in C4-2 compared to LNCaP cells. (A) LNCaP cells were pre-treated for 3 hr with vehicle (V) or the indicated concentrations of inhibitors. The cells were then treated with vehicle or phorbol 12, 13-dibutyrate (PDB), lysed and the lysates normalized for Ran expression. Aliquots of the normalized lysates were electrophoresed and immunoblotted. (B) LNCaP and C4-2 cells were treated with vehicle or EGF, lysed and the lysates normalized and immunoblotted. (C) LNCaP and C4-2 cells were treated with vehicle or EGF, lysed and the endogenous RSK2 immunoprecipitated (IP). An in vitro kinase assay was performed with the RSK2 IP (12). Aliquots of the IP were also electrophoresed and immunoblotted. The specific activity was determined by dividing the kinase data by the relative amounts of RSK2 determined by densitometry of the immunoblots.

Fig. 12 Expression of phospho-p140, RSK1 and RSK2 in human prostate cancer (PC) and normal (N) human prostate. Human tissue was obtained from the Tissue Procurement Facility at the Univ. Virginia. The tissue was finely ground in the presence of liquid N2 and then denatured in lysis buffer. The lysates were normalized to Ran expression and the normalized aliquots were electrophoresed and immunoblotted. (* tissue adjacent to tumor)

Fig. 13 SL0101 inhibits the proliferation of cancer cells but not normal cells. (A) MCF-7 and MCF-10A cells and (B) LNCaP cells were treated with vehicle or 50 M SL0101 or U0126 and a proliferation assay using CellTiter-Glo Luminescent Cell Viability Assay (Promega) was performed 44 hrs after treatment. The data are expressed relative to time 0.

Fig. 14 Rsk1 and Rsk2 are overexpressed in a number of human breast and prostate cancer tissues. (14A) Normal (N) and cancerous breast tissue (LC, lobular carcinoma; MC, mucinous carcinoma; A, adenocarcinomas) was finely ground in the presence of liquid N2 and lysed in SDS-loading buffer. The lysates were normalized to Ran and immunoblotted as shown. This selection is a representative sample of our 26 observations to date. (14B) abbreviations are as follows: Normal (N), prostate cancer (PC) and benign hyperplastic prostate (BPH.

Fig. 15 Rsk2 specifically activates ER - and AR-mediated transcription. (A) MCF-7 or (B) LNCaP cells were co-transfected with a luciferase reporter and -galactosidase expression vectors. Additionally, the cells were transfected with either control vector (V) or a vector encoding constitutively active Rsk2 (Rsk2(Y707A)). The cells were treated with either vehicle, 10 nM estradiol or 5 nM R1881 and/or 100 ng/ml EGF. Luciferase and -galactosidase activity were determined and the luciferase data were divided by the -galactosidase activity to control for differences in transfection efficiency. The data were normalized so that, in the vector control, the response to vehicle addition was zero and the response to either estradiol or R18181 was 100. The values are +SEM. *P<0.05 and **P<0.01 (Student's t-test) obtained by comparing the response obtained with the vector control with that obtained with Rsk2(Y707A).

Detailed Description of the Invention

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, the term "purified" and like terms relate to the isolation of a molecule or compound in a form that is substantially free (at least 60% free, preferably 75% free, and most preferably 90% free) from other components normally associated with the molecule or compound in a native environment.

As used herein, the term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water and emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents.

As used herein, an "effective amount" means an amount sufficient to produce a selected effect. For example, an effective amount of an Rsk inhibitor is an amount of the inhibitor sufficient to suppress Rsk activity in a serine/threonine kinase assay.

The general chemical terms used in the description of the compounds of the present invention have their usual meanings. For example, the term "alkyl" by itself or as part of another substituent means a straight or branched aliphatic chain having the stated number of carbon atoms.

The term "halo" includes bromo, chloro, fluoro, and iodo.

The term "haloalkyl" as used herein refers to a alkyl radical bearing at least one halogen substituent, for example, chloromethyl, fluoroethyl or trifluoromethyl and the like.

The term "C_1 -C_n alkyl" wherein n is an integer, as used herein, refers to a branched or linear alkyl group having from one to the specified number of carbon atoms. Typically C_1 -C_6 alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, hexyl and the like.

The term "C_2 -C_n alkenyl" wherein n is an integer, as used herein, represents an olefinically unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one double bond. Examples of such groups include, but are not limited to, 1-propenyl, 2-propenyl, 1,3-butadienyl, 1-butenyl, hexenyl, pentenyl, and the like.

The term "C_2 -C_n alkynyl" wherein n is an integer refers to an unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one triple bond. Examples of such groups include, but are not limited to, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, and the like.

As used herein, the term "optionally substituted" refers to zero to four substituents, wherein the substituents are each independently selected. More preferredly, the term refers to zero to three independently selected substituents.

As used herein the term "aryl" refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like. Aryl groups (including bicyclic aryl groups) can be unsubstituted or substituted with one, two or three substituents independently selected from loweralkyl, haloalkyl, alkoxy, amino, alkylamino, dialkylamino, hydroxy, halo, and nitro. Substituted aryl includes aryl compounds having one or two C_1 -C_6 alkyl, halo or amino substituents. The term (alkyl)aryl refers to any aryl group which is attached to the parent moiety via the alkyl group.

The term "C_3 -C_n cycloalkyl" wherein n = 4-8, represents cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl.

The term "heterocyclic group" refers to a C_3-C_8 cycloalkyl group containing from one to three heteroatoms wherein the heteroatoms are selected from the group consisting of oxygen, sulfur, and nitrogen.

The term "bicyclic" represents either an unsaturated or saturated stable 7- to 12-membered bridged or fused bicyclic carbon ring. The bicyclic ring may be attached at any carbon atom which affords a stable structure. The term includes, but is not limited to, naphthyl, dicyclohexyl, dicyclohexenyl, and the like.

The term "lower alkyl" as used herein refers to branched or straight chain alkyl groups comprising one to eight carbon atoms, including methyl, ethyl, propyl, isopropyl, n-butyl, t-butyl, neopentyl and the like.

The term, "parenteral" means not through the alimentary canal but by some other route such as subcutaneous, intramuscular, intraspinal, or intravenous.

As used herein, the term "treating" includes alleviating the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms.

As used herein, the term "RSK inhibitor" includes any compound or condition that specifically inhibits RSK kinase activity. Such inhibitory effects may result from directly or indirectly interfering with the protein's ability to phosphorylate its substrate, or may result from inhibiting the expression (transcription and/or translation) of RSK.

The Invention

p90 Ribosomal S6 Kinase (RSK) is a serine/threonine kinase that is a downstream component of the Mitogen-activated Protein Kinase (MAPK) signaling pathway and is activated by MAPK. MAPK is activated by several diverse signals under normal conditions and is hyperactive in many diseased states due to improper regulation of the signaling pathway. Activation of MAPK results in increased RSK catalytic activity. However, defining the physiological function of RSK activation under normal and diseased conditions has been difficult because there are no RSK-specific inhibitors.

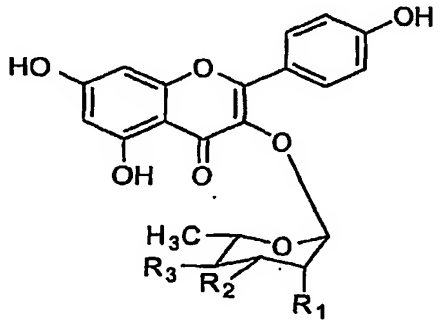
As described herein an Rsk-specific inhibitor has now been identified from botanical extracts using novel high throughput screening (HTS) Enzyme-Linked Immunosorbent Assays (ELISA) that produce luminescence as a measure of substrate phosphorylation. To discriminate extracts containing Ser/Thr kinase inhibitors from those containing nuisance compounds, a dual screen of the extracts was performed using either a constitutively active mutant of isoform 2 of Rsk (Rsk2) or the catalytic domain of the tyrosine kinase, Focal Adhesion Kinase (FAK). Of 1500 botanical extracts assayed, only one, from *Forsteronia refracta*, a member of the Dogbane family, inhibited Rsk2 without inhibiting FAK (Fig. 6a). To determine whether the extract contained a general Ser/Thr kinase inhibitor, activities of the archetypal Ser/Thr kinase, protein kinase A (PKA) and of two kinases most closely related to Rsk2, p70 S6K and Msk1, were measured in the presence of varying amounts of extract (Fig. 6b). Amounts of extract that inhibited Rsk2 activity by 90% did not inhibit PKA, p70 S6K or Msk1 to a greater extent than FAK. Thus, the *F. refracta* extract contains an inhibitor with remarkable specificity for Rsk2 relative to these other AGC kinase family members.

The effect of extract on Rsk2 activity was measured in the presence of increasing concentrations of ATP (Fig. 6c). Extract did not reduce the maximal velocity of the reaction, but increased the concentration of ATP required to support half-maximal velocity by approximately 20-fold. Thus, the mechanism of Rsk inhibition by the extract is competitive with respect to ATP.

One embodiment of the present invention is directed to compositions comprising an extract from the plant *Forsteronia refracta* (a member of the dogbane family found in the South

American rain forest) that has activity as an RSK specific inhibitor. More particularly the present invention is directed to an alcohol, preferably methanol, extract of wood stem and/or stem bark of *Forsteronia refracta*. In one embodiment the present invention is directed to a composition comprising one or more purified flavonoids extracted from the tissues of *Forsteronia refracta*. These compositions can be further combined with pharmaceutically acceptable carriers and other therapeutic compounds to provide therapeutic pharmaceutical compositions for treating a wide range of diseases that are associated with inappropriate Rsk activity.

In one embodiment the present invention is directed to a composition comprising a compound of the general formula:



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wherein R_1, R_2 and R_3 are independently selected from the group consisting of H, hydroxy -$OCOR_4$, -COR_4 and C_1-C_4 alkoxy, and R_4 is H or C_1-C_4 alkyl. In one embodiment R_1 and R_2 are independently selected from the group consisting of hydroxy and -OAc and R_3 is -OAc. The compositions may further comprise a pharmaceutically acceptable carrier.

The individual compounds, SL0101-1, SL0101-2 and SL0101-3 (see Fig. 1), are collectively referred to as SL0101. SL0101-1, SL0101-2 and SL0101-3 are ATP-mimetics (see Examples 1 & 2) that inhibit RSK *in vitro* with IC_{50}s of 90 nM, 580 nM and 190 nM, respectively (Fig. 2). However, they do not inhibit the evolutionarily related p70 S6 kinase and Mitogen- and Stress-activated Protein Kinase (MSK). In addition, they do not inhibit the prototypical serine/threonine kinase Protein Kinase A or the tyrosine kinase Focal Adhesion Kinase (FAK) (Fig. 3).

Specific inhibition of RSK *in situ* was determined by incubation of MCF-7 cells in the presence or absence of increasing concentrations of extract fraction enriched in SL0101 prior to stimulation of the MAPK pathway with phorbol dibutyrate (PDB). The presence of the RSK inhibitor eliminated phosphorylation of the RSK substrates Estrogen Receptor alpha (ERa) and pp140 as determined using phospho-specific antibodies developed using the RSK phosphorylation site in the ERa as the antigen (Fig. 4). However, the inhibitor did not alter phosphorylation of RSK by MAPK as indicated by the generation of RSK with reduced mobility observed following SDS-PAGE. The inhibitor did not influence activation of MAPK by the MAPK Kinase, MEK as determined by the phospho-specific antibody recognizing active MAPK. Therefore, SL0101 did not inhibit the catalytic activity of Protein Kinase C (PKC), RAF, MEK, or MAPK because these kinases are essential to cause phosphorylation of RSK in cells stimulated with PDB. Thus, SL0101 is a RSK-specific inhibitor *in situ* as well as *in vitro* and can be used as an investigative tool for defining the function of RSK *in situ*.

Inhibition of RSK inhibits proliferation of Ha-ras-transformed NIH/3T3 cells without influencing the proliferation rate of non-transformed NIH/3T3 cells. Ha-ras-transformed NIH/3T3 cells or parental NIH/3T3 cells were incubated in the presence of vehicle, 50 mM SL0101, or 50 mM PD 98059, a MEK-specific inhibitor. The presence of SL0101 inhibits Ha-ras-transformed NIH/3T3 cell proliferation over a 48 hour time course, even in the presence of 10 % fetal calf serum (Fig. 5). However, SL0101 had little influence on the rate of parental NIH/3T3 proliferation compared to that observed in the presence of vehicle. An influence on the proliferation rate by the MEK inhibitor, PD 98059 was observed only when cells were incubated in the presence of low concentrations of fetal calf serum (0.1 - 1 %) (data not shown).

Thus, RSK-specific inhibitors have been shown to inhibits proliferation of a transformed cell without substantially altering the rate of non-transformed cell growth. Therefore, SL0101 is not toxic to non-transformed cells. These data suggest that RSK-specific inhibitors can be used as an anti-cancer therapy by abolishing the growth of malignant tumors without toxic effects on the normal tissues. In addition, because SL0101 inhibits RSK specifically *in situ* without toxic effects, RSK inhibitors can be also be used as therapeutic interventions in non-terminal diseased states such as epilepsy in which the MAPK signaling pathway is improperly regulated. In

accordance with one embodiment, the RSK-specific inhibitors of the present invention are used to treat cancer and neurological disorders. "Treating" as used herein includes administering therapy to prevent or cure the disease (for example, for cancer this includes inhibiting tumor initiation and progression), as well as alleviating the symptoms associated with the disease/disorder.

In accordance with one embodiment of the present invention a method for inhibiting Rsk kinase activity in a subject is provided, as a means of treating an illness associated with inappropriate Rsk activity. For example such a method can be used to treat a patient diagnosed with a neoplastic disease. In accordance with one embodiment the method comprises the steps of administering to said subject a therapeutically-effective amount of a composition comprising an extract from the tissues of *Forsteronia refracta*. Applicants have demonstrated that Rsk inhibitors are effective in preventing the proliferation of prostate and breast cancer cells. The active natural compounds isolated from the tissues of *Forsteronia refracta* can be combined, or used in conjunction with, other known anti-cancer agents such as chemotherapeutics or radiation treatments to effectively treat cancer patients.

The Rsk inhibitory compositions of the present invention can be administered either orally or parenterally. In one embodiment Rsk inhibiting composition is administered locally by injection or implantable time release device. When administered orally, the compounds can be administered as a liquid solution, powder, tablet, capsule or lozenge. The compounds can be used in combination with one or more conventional pharmaceutical additive or excipients used in the preparation of tablets, capsules, lozenges and other orally administrable forms. When administered parenterally, and more preferably by intravenous injection, the derivatives of the present invention can be admixed with saline solutions and/or conventional IV solutions.

One embodiment of the present invention is directed to pharmaceutical compositions comprising an Rsk inhibitory extract of and a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier can be selected from among the group consisting of excipients, disintegrating agents, binders and lubricating agents. In a further aspect, the present invention provides a pharmaceutical composition comprising a flavonoid of the general formula I as defined above and a pharmaceutically acceptable carrier or diluent. The amount of the

pharmaceutical agent suitable for administration will be in accordance with standard clinical practice.

Example 1

Screening protocol

A high throughput screening (HTS) Enzyme-Linked Immunosorbent Assays (ELISAs) that can be used to screen for inhibitors of the various classes of kinases has been developed. These ELISAs can be used to obtain a robust signal-to-noise level for each of the various classes: RSK represents the class of Ser/Thr kinases, focal adhesion kinase (FAK) represents the Tyr kinases and extracellular-signal regulated kinase 2 (ERK2) represents the Pro-directed Ser kinases. The ELISAs utilize horseradish peroxidase (HRP)-conjugated phosphospecific antibodies or phosphospecific antibodies in combination with HRP-conjugated secondary antibodies. The Z' factor of an assay is a statistical characteristic of the quality of the assay with respect to the dynamic range and data variation of the signal measurements. A Z' factor equal to 1 represents the ideal assay with no background and no deviation of signal, whereas a $Z' \leq 0.5$ indicates that the signal window is small to non-existent. The Z' of the HTS ELISAs that we developed is ~0.8, substantially higher than other HTS assays developed for the identification of kinase inhibitors. We have successfully used this HTS ELISAs to screen a botanical extract library. Each plate in the screen contained 80 extracts as well as controls. These controls ensure that there was no plate-to-plate variation in the screen.

Using the GENESIS Workstation 150 our throughput rate is ~2000 assays/day. Therefore, screening in duplicate the NCI synthetic set of compounds, which consists of 1990 compounds in the Diversity set, 879 compounds in the Mechanistic set and 57 compounds in the Challenge set, will take ~ 3 days. Data analysis will take ~ one week. However, a single screen of the entire collection of natural products extracts of 140,000 in duplicate can be accomplished in ~ 140 days. Data analysis will take several months.

Example 2

Isolation of Rsk inhibitors

To identify a Rsk-specific inhibitor from botanical extracts, a novel high throughput screening (HTS) Enzyme-Linked Immunosorbent Assays (ELISA) that produce luminescence as a measure of substrate phosphorylation was used. To discriminate extracts containing Ser/Thr kinase inhibitors from those containing nuisance compounds, a dual screen of the extracts was performed using either a constitutively active mutant of isoform 2 of Rsk (Rsk2) or the catalytic domain of the tyrosine kinase, Focal Adhesion Kinase (FAK). Of 1500 botanical extracts assayed, only one, from *Forsteronia refracta*, a member of the Dogbane family, inhibited Rsk2 without inhibiting FAK (Fig. 1a).

Purification and structure determination of three inhibitors isolated in methanolic extracts from the plant *Forsteronia refracta* have been completed. The methanolic extract from wood stem and stem bark of *Forsteronia refracta* was applied to a polyamide 6S column, which was washed successively with H_2O, 1:1 H_2O- MeOH, 9:1 CH_2Cl_2-MeOH, 1:1 CH_2Cl_2-MeOH and 9:1 MeOH-NH_4OH to afford five fractions. The 9:1 CH_2Cl_2-MeOH and 1:1 CH_2Cl_2-MeOH fractions showed stronger inhibition of RSK than starting material. The 1:1 CH_2Cl_2-MeOH fraction was further fractionated on a diol gel column. The column was eluted successively with CH_2Cl_2, 99:1 CH_2Cl_2-MeOH, 95:5 CH_2Cl_2-MeOH, 90:10 CH_2Cl_2-MeOH and MeOH to give five fractions. Among these, the 95:5 CH_2Cl_2-MeOH, 90:10 CH_2Cl_2-MeOH and MeOH fractions showed the same or stronger activity than the starting material. The 95:5 CH_2Cl_2-MeOH fraction was fractionated repeatedly on a C_{18} reverse phase HPLC column (250′10 mm); elution was carried out with 65:35 MeOH-H_2O and UV detection was at 265 nm. Two compounds, SL0101-1 and SL0101-2 were obtained as amorphous pale yellow powders. On the basis of its ^1H NMR spectrum and positive APCI-MS, SL0101-1 was found to be kaempferol 3-a-L-(3",4"-diacetyl) rhamnopyranoside and SL0101-2 was proved to be kaempferol 3-a-L- (2",4"-diacetyl) rhamnopyranoside (See Fig. 1).

The 90:10 CH_2Cl_2-MeOH fraction from above diol column was also fractionated repeatedly on a C_{18} reverse phase HPLC column using 45:55 H_2O-MeOH as the eluant and UV detection at 275 nm. The active constituent, SL0101-3, was obtained as an amorphous powder. On the basis

of its ^{1}H NMR and ^{13}C NMR data, the compound was found to be kaempferol-3-a-L- (4"-acetyl) rhamnopyranoside (SLO101-3).

Structural determination identified the inhibitor, as a kaempferol glycoside (Fig. 6e).. The *in vitro* IC50 was determined to be less than 100 nM (Figs. 6d and 6e), whereas the IC50 of SL0101-1 kaempferol, the flavonoid constituent of SL0101-1 was determined to be 15 M for Rsk (Fig. 6d). Therefore, the rhamnose moiety of SL0101-1 greatly increases the affinity for Rsk. Purified SL0101-1 is specific for inhibition of Rsk activity compared to p70 S6K and Msk1 and is competitive with respect to ATP (Fig. 6f).

Rsk contains two non-related kinase domains in a single polypeptide chain. The NTKD is mostly closely related to p70 S6K whereas the carboxyl-terminal kinase domain (CTKD) is most similar to the calmodulin-dependent protein kinases. Regulation of Rsk2 is complex and requires a cascade of phosphorylations resulting from the actions of MAPK, the CTKD of Rsk itself, and 3-phosphoinositide-dependent protein kinase-1. The NTKD phosphorylates exogenous substrates whereas the only known function of the CTKD is autophosphorylation. Inhibition of Rsk by SL0101-1 could occur through interaction with either kinase domain. To determine the domain inhibited by SL0101-1, the ability of SL0101-1 to inhibit full-length or a truncation mutant of Rsk2 containing only the NTKD (Rsk2(1-389)) was compared (Fig. 7a). SL0101-1 potently inhibited the isolated Rsk2 NTKD, indicating that inhibition of Rsk occurs through competition with ATP for the nucleotide-binding site of the NTKD.

Alignment of residues forming the ATP-binding pocket of Rsk with those of p70 S6K, Msk1 and PKA revealed a difference in contacts to the adenosine ring. Indeed, the sequence 145LILDFLRGGDLFT157, referred to as the adenosine-interacting loop (AIL), is unique to the Rsk family (Fig. 7b). To examine the importance of this region in determining SL0101-1 specificity, a mutant Rsk2 was created in which the p70 S6K AIL (147LILEYLSGGELFM159) replaced that of Rsk2 (Rsk2-AILmutant). SL0101-1 was ~ 3-fold less effective in inhibiting the mutant in comparison to wild type Rsk2 (Fig. 7c). Therefore, the unique adenosine-interacting loop of Rsk is a major determinant for SL0101-1 binding. However, the mutation did not completely abolish inhibition by SL0101-1, indicating the presence of additional points of contact. To determine whether the unique adenosine-interacting loop is sufficient for SL0101-1

specificity the isozyme specificity of SL0101-1 was examined. The primary structure of the NTKDs of the Rsk isoforms 1-3 are highly related, sharing 87% identity and each contain the unique adenosine-interacting loop. Remarkably, however, although SL0101-1 potently reduced Rsk1 and Rsk2 activity, the Rsk3 activity was only partially inhibited (Fig. 7c). Thus, the adenosine interacting loop is necessary but not sufficient for conferring SL0101-1 specificity and the Rsk1 and Rsk2 isoforms must have additional contact points that increase the affinity for SL0101-1. These results further attest to the remarkable specificity of SL0101-1.

Methods

Polyamide 6S (pour density 0.25 g/mL, a product of Riedel-de Haen, Germany) was obtained from Crescent Chemical Co. Lichroprep diol (40-63 m) is a product from EM Industries, Inc. A Kromasil C18 reverse phase column (250 10 mm, 5 m) for HPLC was obtained from Higgins Analytical Inc. 1H NMR spectra were measured on General Electric QE 300, GN-300 NMR or Varian unity INOVA-500 spectrometers. Mass spectra were recorded on a Finnigan MAT 4600 mass spectrometer.

Wood stem and stem bark of Forsteronia refracta was soaked three times with methanol at room temperature. The resulting methanol solutions were combined and concentrated under diminished pressure to afford the crude extract. The crude extract (888 mg) was applied to a 40-g polyamide 6S column, which was washed successively with 150 mL each of H2O, 1:1 H2O- MeOH, 9:1 CH2Cl2-MeOH, 1:1 CH2Cl2-MeOH and 9:1 MeOH-NH4OH to afford five fractions. The 1:1 CH2Cl2-MeOH fraction (126.7 mg) showed stronger inhibition of Rsk than the starting original extract. The 1:1 CH2Cl2- MeOH fraction was further fractionated on a 30-g diol gel column. The column was washed, respectively, with 150 mL each of CH2Cl2, 99:1 CH2Cl2-MeOH, 95:5 CH2Cl2-MeOH, 90:10 CH2Cl2-MeOH and MeOH to give five fractions. Among these, the 95:5 CH2Cl2-MeOH (38.1 mg) fraction showed the same or stronger activity than the starting material. The 95:5 CH2Cl2-MeOH fraction (4 mg) was fractionated repeatedly on a C18 reverse phase HPLC column (250 10 mm); with elution was 65:35 MeOH-H2O at a flow rate of 3 mL/min, with UV detection at 265 nm. SL0101-1 (2 mg) was obtained as an

amorphous pale yellow powder. From the 1H NMR and positive APCI-MS, SL0101-1 was confirmed to be kaempferol 3- -L-(3",4"-diacetyl) rhamnopyranoside

Example 3

***In vivo* activity of Rsk inhibitors**

To determine whether SL0101-1 inhibits Rsk in intact cells, phosphorylation of p140, a Rsk substrate of unknown function, was examined in a human breast cancer cell line, MCF-7. Pre-incubation of cells with 100 M SL0101-1 abrogates phorbol dibutyrate (PDB)-induced p140 phosphorylation as does 50 M U0126, a MEK inhibitor (Fig. 8a). SL0101-1 does not effect the phosphorylation of Rsk2, as indicated by the reduced electrophoretic mobility of Rsk2, nor the activation of MAPK, as detected by the anti-active MAPK antibody (Fig. 8a). Therefore, SL0101-1 does not inhibit upstream kinases necessary for PDB-stimulated Rsk phosphorylation, namely MAPK, MEK, Raf and PKC. These data indicate that SL0101-1 is an effective and specific Rsk inhibitor in intact cells.

The importance of MAPK to proliferation and oncogenesis is well established. However, the role that Rsk plays in these processes has not been examined. Therefore, the effect of SL0101-1 on proliferation of Ha-Ras transformed NIH/3T3 cells and the parental cell line was determined. SL0101-1 decreased the growth rate of the transformed cells but had little effect on proliferation of the parental line (Fig. 8b). SL0101-1 produced striking morphology changes in the transformed cells but not in the parental cell line. The vehicle control treated Ha-Ras transformed cells were elongated whereas in response to SL0101-1 the cells became much larger and flatter, appearing more like the parental cells, or like Ha-Ras transformed cells treated with U0126 (Fig. 8c). Removal of SL0101-1 resulted in growth of the transformed cells and a reversion to their elongated phenotype (Fig. 8d). These results demonstrate that SL0101-1 can penetrate intact cells, but is not toxic and preferentially inhibits the growth of oncogene-transformed cells compared to the parental cells.

Whether or not SL0101-1 could inhibit the growth rate of MCF-7 cells, which are more representative of human cancers than the Ha-Ras transformed cell line, was also investigated. Remarkably, SL0101-1 inhibited proliferation of MCF-7 cells but had no effect on the growth of

the normal breast cell line, MCF-10A (Fig. 8e), even though SL0101-1 prevented the PDB-induced p140 phosphorylation in MCF-10A cells (Fig. 8a).

Reduction of Rsk1 and Rsk2 levels was also accomplished using short, interfering RNAs (siRNA), and a combination of siRNAs to both Rsk1 and Rsk2 was effective in reducing MCF-7 proliferation (Fig. 8f). The siRNAs were not as effective at inhibiting growth as SL0101-1, however Rsk1 and Rsk2 expression was not completely eliminated and only about 70% of the cells were transfected. Nonetheless, these results strongly support observations that both Rsk1 and Rsk2 are important in MCF-7 proliferation.

As further support of the specificity of SL0101-1 action, U0126, the MEK inhibitor, halted proliferation of both MCF-7 and MCF-10A cells (Fig. 9a). Ro 318220 (500 nM), a potent but non-specific PKC inhibitor, which inhibits Rsk as well as a number of other AGC kinase family members also attenuated proliferation of both MCF-7 and MCF-10A cells. Moreover, kaempferol, the flavonoid constituent of SL0101-1 slows growth of MCF-10A and MCF-7 cells to the same extent. Therefore, unlike the action of these other kinase inhibitors, SL0101-1 selectively halts proliferation of cancer cells without affecting normal cells.

The involvement of Rsk in breast cancer has not previously been examined. It has been suggested in the literature that among the numerous events involved in tumor initiation and progression is an increased reliance on the signaling pathway for which regulation has been compromised as well as the dormancy of alternative signaling pathways. Thus it is possible that the growth of MCF-7 cells have become dependent on the Rsk pathway rendering these cells susceptible to inhibition by SL0101-1. The growth of MCF-10A cells would not be inhibited by SL0101-1 because all signaling pathways regulating proliferation are intact providing numerous mechanisms for circumventing inhibition of a single signaling event. Interestingly, MCF-7 cells overexpressed Rsk2 in comparison to MCF-10A and ~ 50% of breast cancers have elevated levels of either Rsk1 or Rsk2 compared to normal tissue (Figs. 8a and 9b). It is anticipated that the growth of tumors, such as breast cancer, that overexpress Rsk will be susceptible to inhibition by SL0101-1 and that Rsk-specific inhibitors, such as SL0101-1, may find wide spread use as chemotherapeutic agents. As reported herein, SL0101-1 inhibited proliferation of MCF-7 cells but did not cause cell death (Fig. 8e). However, SL0101-1 when used in combination with

activation of the stress pathways, e.g. by serum deprivation, significantly reduced cell viability compared to vehicle control (Fig. 9c). Thus, Rsk inhibitors may be most effective when combined with other anti-cancer therapies. Rsk inhibitors may also be effective at inhibiting the growth of cancers in which the MAPK pathway is overactive as indicated by the result that SL0101-1 inhibited the proliferation of Ha-Ras transformed cells but not the parental cells.

Our studies have uncovered an unexpected link between Rsk activity and tumor cell proliferation revealing the value of Rsk as a novel drug target. Additionally, we have identified the first Rsk-specific inhibitor, SL0101-1, which will provide a powerful new tool for analyzing Rsk function in a variety of biological systems.

Methods.

Kinase Assays. Glutathione-S-transferase (GST)-fusion protein (1 g) containing the sequence -RRRLASTNDKG (for serine/threonine kinase assays) or -VSVSETDDYAEIIDEEDTFT (for tyrosine kinase assays) was adsorbed in the wells of LumiNunc 96-well polystyrene plates (MaxiSorp surface treatment). The wells were blocked with sterile 3% tryptone in phosphate buffered saline and stored at 4°C for up to 6 months. Kinase (5 nM) in 70 l of kinase buffer (5 mM -glycerophosphate pH 7.4, 25 mM HEPES pH 7.4, 1.5 mM DTT, 30 mM MgCl2, 0.15 M NaCl) was dispensed into each well. Compound at indicated concentrations or vehicle was added and reactions were initiated by the addition of 30 l of ATP for a final ATP concentration of 10 M unless indicated otherwise. Reactions were terminated after 10 to 45 min by addition of 75 l 500 mM EDTA, pH 7.5. All assays measured the initial velocity of reaction. After extensive washing of wells, polyclonal phosphospecific antibody developed against the phosphopeptide and HRP-conjugated anti-rabbit antibody (211-035-109, Jackson ImmunoResearch Laboratories) were used to detect serine phosphorylation of the substrate. HRP-conjugated anti-phospho-tyrosine antibody (RC20, BD Transduction Laboratories) was used for phospho-tyrosine detection. His-tagged active Rsk and FAK were expressed in Sf9 cells and purified using NiNTA resin (Qiagen). Baculovirus was prepared using the Bac-to-Bac® baculovirus expression system (Invitrogen). PKA was bacterially expressed and activated as described (Anal. Biochem. 245, 115-122 (1997)). Active Msk1 and p70 S6 kinase was

purchased from Upstate Biotechnology. Immunoprecipitation and kinase assays were performed as previously described5 using the immobilized GST-fusion proteins and ELISAs as above.

Cell Culture. For proliferation studies cells were seeded at 2500 to 5000 cells per well in 96 well tissue culture plates in the appropriate medium as described by American Type Culture Collection. After 24 hr, the medium was replaced with medium containing compound or vehicle as indicated. Cell viability was measured at indicated time points using CellTiter-Glo™ assay reagent (Promega) according to manufacturer's protocol. For *in vivo* inhibition studies, cells were seeded at 2.5 X105 cells/well in 12 well cell culture clusters. After 24 hr, the cells were serum starved for 24 hr then incubated with compound or vehicle for 3 hr prior to a 30 min PDB stimulation. Cells were lysed as previously described(J. Biol. Chem. 273, 13317-13323 (1998)). The lysates were normalized for total protein, electrophoresed and immunoblotted. For cell imaging, Ha-Ras-transformed NIH/3T3 cells were seeded on LABTEK II chamber slides (Nalge) at a density of 1 X104 cells/well. After 24 hr, fresh medium was added the indicated compounds or vehicle. Images were taken 48 hr after treatment at a magnification of 20X.

Gene Silencing. Custom oligonucleotides to Rsk1 and Rsk2 mRNA (Dharmacon Research Inc.) (sequence available upon request) and TransIT-TKO® siRNA Tranfection Reagent (MIR2150, Mirus Corporation) were used for the gene silencing studies. MCF-7 cells were seeded at a density of 1.25X104 cells per well in 24 well cell culture clusters. After 24 hr, fresh medium was added containing 25 nM oligonucleotide and transfection reagent according to manufacturer's protocol. The transfection medium was replaced after 24 hr. Cells were incubated for an additional 48 hr prior to cell viability measurement.

Breast tissue analysis. Frozen tissue samples were ground using mortar and pestle under liquid nitrogen. Ground tissue was added to heated 2-X SDS loading buffer and boiled for 3 min. Protein concentration of lysates was measured and lysates were electrophoresed on SDS-PAGE and immunoblotted.

Example 4

Rsk Inhibitors inhibit proliferation of Prostate Cancer Cell Line

Prostate cancer is the second most common cancer in men and approximately one in six men will be diagnosed with the disease. Early stage prostate cancer is frequently dependent on the hormone, androgen. Androgen action is mediated through interaction with the androgen receptor, a member of the superfamily of ligand-activated transcription factors. Inhibition of androgen receptor activity by pharmacological or surgical interventions that reduce androgen concentration can result in prostate tumor regression. However, with relatively high frequency the tumors become androgen-independent, which often leads to a fatal outcome. Treatment options are confined to conventional chemotherapy because of the lack of specific drug targets associated with androgen-independent prostate cancer. Thus, elucidation of the mechanisms that result in the transition of prostate cancer from an androgen-dependent to androgen-independent state will greatly facilitate the identification of more effective therapies.

An increase in mitogen-activated protein kinase (MAPK) activity has been correlated to prostate cancer progression in human tumors. This enhanced activity is most likely due to the increase in growth factors and receptors that are known to occur. Activation of growth factor receptors enhance MAPK activity via a kinase cascade that is regulated by the small GTP-binding protein, p21Ras. The family of p90 ribosomal S6 kinases (RSKs), which are Ser/Thr protein kinases, function as downstream effectors of MAPK. The biological actions of the RSKs are not well characterized partly because until recently there were no known inhibitors of RSK that did not also inhibit MAPK activity.

The first RSK-specific inhibitor, SL0101 has now been isolated. SL0101 inhibits the proliferation of the breast cancer cell line, MCF-7, with an efficacy that parallels its ability to suppress RSK activity *in vivo*. Extraordinarily, SL0101 does not prevent the proliferation of a normal breast cell line, MCF-10A, even though SL0101-induced inhibition of a RSK substrate is observed in these cells. This exciting differential effect is not mimicked by inhibition of the upstream activator of MAPK, using U0126, which inhibits proliferation of both cell types. Furthermore, in NIH 3T3 fibroblasts, SL0101 reduces the growth of a Ha-Ras-transformed line but not of the untransformed parental cells. It is believed that SL0101 does not inhibit the

growth of untransformed cells because multiple pathways regulate their proliferation and therefore, inhibition of any single signaling event does not influence proliferation. In contrast, transformed cells are inhibited by SL0101 because they preferentially depend on the RSK pathway to regulate proliferation. These results provide the first demonstration that the RSK family through the regulation of its downstream effectors is involved in the control of cancer cell proliferation. Relatively few downstream effectors of RSK have been identified. However, RSK is known to phosphorylate and regulate the activity of a number of transcription factors, the pro-apoptotic protein, BAD, and the mitotic checkpoint kinase, BUB1. Determining which RSK substrates play a key role in cancer cell proliferation will undoubtedly lead to the discovery of novel drug targets for cancer therapy.

Because activation of the MAPK pathway appears to be correlated with prostate cancer progression, the ability of SL0101 to inhibit the proliferation of the androgen-dependent human prostate line, LNCaP and the androgen-independent line, C4-2 was tested. The C4-2 line was derived from the LNCaP line by serial passage in castrated mice and was not explicitly selected for changes in the MAPK signal transduction pathway. The LNCaP line is a model for early stage and the C4-2 line is a model for late stage prostate cancer. These lines are a good model system in which to study the importance of RSK function in prostate cancer progression because they have been very well characterized and have a single lineage. SL0101 completely inhibits the proliferation of LNCaP cells but only partially inhibits the proliferation of C4-2 cells (Fig. 10). This result suggests that the LNCaP line has become primarily dependent on RSK activity for growth, but that, during the process of becoming androgen-independent the C4-2 cells have bypassed this requirement.

To investigate the differences in the RSK signal transduction pathway between the LNCaP and the C4-2 lines, a phosphospecific antibody to a RSK phosphorylation motif (RPM) was produced. Only a few downstream effectors of RSK have been identified and therefore, applicants anticipated that an anti-RPM antibody would be a very effective tool for identifying novel RSK substrates *in vivo*. An anti-RPM antibody has previously been reported that recognizes the RSK substrate, p140, a protein of unknown function. In agreement with these results SL0101 was observed to decrease the phosphorylation of p140 with an efficacy that

paralleled its ability to inhibit LNCaP proliferation (Fig. 13A). All our lysates were normalized to each other using an anti-Ran antibody. Ran is used for normalization based on the observations that it is a general housekeeping protein, the activity or expression levels of which are not known to vary in any disease state.

Interestingly, in C4-2 cells, activation of RSK by epidermal growth factor (EGF) did not increase the phosphorylation of p140 as in LNCaP cells, although, as shown by the anti-active MAPK immunoblot, MAPK was activated to the same extent in both lines. However, the endogenous RSK in C4-2 cells has ~ 1/3rd the specific activity as that in LNCaP cells (Fig. 13C). These results show that RSK activity has been compromised in C4-2 cells, which results in the disruption of the RSK signal transduction pathway, as seen by the absence of p140 phosphorylation. Thus, it is likely that SL0101 is unable to effectively inhibit C4-2 proliferation due to the inability of RSK to regulate its effectors in these cells. It may be that this disruption of the RSK signal transduction pathway is involved in the progression from androgen-dependence to androgen-independence. It may be possible to bypass this disruption in RSK signaling with small-molecule modulators of RSK downstream effectors and thereby resume normal regulation of the pathway.

To determine whether disruption of RSK signal transduction pathway is physiologically significant normal human prostate and prostate cancer tissue were analyzed for expression of the RSK isoforms 1 and 2 (RSK1 and RSK2) and the phosphorylation of p140. The cancer tissues have higher levels of RSK1 and RSK2 expression than the normal tissues with the exception of one normal sample, which had a high level of RSK1 expression. However, this sample was removed from tissue that was adjacent to cancerous tissue (Fig. 13B). Interestingly, phosphorylation of p140 could be detected in normal prostate tissues except for the one normal tissue that also contained a higher level of RSK1 expression. Under the electrophoretic conditions used in this experiment the phosphorylated p140 migrates as a doublet. The cancerous tissue was obtained from tumors with Gleason scores>7, which indicates that the samples are of advanced prostate cancers.

Malignant transformation and progression in human cancers are frequently associated with over-abundance or increased activity of proteins that are involved in normal cellular

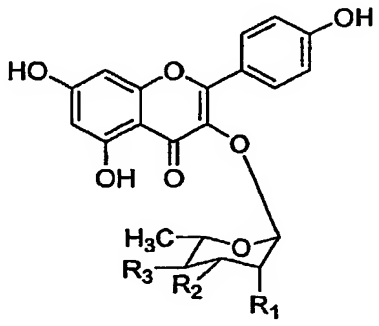
processes. We have found that the Rsks are overexpressed in many human breast and prostate cancers, as compared to normal breast and prostate tissue. Lysates were made from the various samples and normalized to each other using an anti-ran antibody. Ran was used for normalization based on the observations that it is a general housekeeping protein whose activity or expression levels are not known to vary in any disease state. We examined Rsk1 and Rsk2 expression in 22 breast cancer samples and 4 normal samples and found that > 50% of the breast cancer tissues have higher Rsk expression than the normal samples (Fig. 14A). Rsk1 and Rsk2 expression was also examined in 4 prostate cancer, 5 normal and 5 benign hyperplastic (BPH) samples. In general, the cancer tissues have higher levels of Rsk expression than the normal and BPH tissue with the exception of one normal sample. However, this sample was removed from tissue that was adjacent to cancerous tissue (Fig. 13B). The breast and prostate lysates were also immunoblotted with anti-pan ERK antibody, which recognizes both the active and inactive forms of p42 and p44 MAPK (Fig. 14). The relative levels of p42 and p44 MAPK varied considerably between the samples but did not correlate with the extent of Rsk overexpression. Thus Rsk overexpression is not merely a reflection of overexpression of various members of the MAPK pathway. These preliminary results are encouraging because they suggest that both Rsk1 and Rsk2 activity may be higher in human breast and prostate cancer tumors than in normal human breast tissue. These results support our hypothesis that Rsk would be a good drug target for breast and prostate cancer, and possibly for other cancer types.

We have also found that overexpression of the isoform 2 of the Rsk family (Rsk2) enhances the transcriptional activity of the estrogen receptor (ER) and the androgen receptor (AR). We generated a constitutively active mutant of Rsk2 to allow us to study the role of Rsk2 in ER -mediated transcription in the absence of active MAPK. Rsk2 enhanced both ligand-dependent and -independent ER -mediated transcription in MCF-7 cells, a human breast cancer cell line (Fig.). Additionally, we have recently found that Rsk2 enhanced the ligand-dependent and -independent transcription of AR-mediated transcription in LNCaP cells, a human prostate cancer cell line (Fig. 15B). These results are significant because they suggest that the enhanced Rsk expression we observed in breast and prostate cancer cells may increase ER or AR

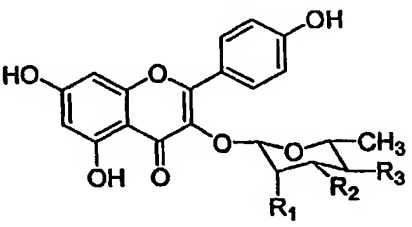
transcriptional activity. Increased activities of the ER and AR are known to be important in the etiology of some breast and prostate cancers, respectively.

Claims

1. A method of specifically inhibiting Rsk activity, said method comprising the step of contacting a Rsk enzyme with a compound having the structure:



wherein R_1 is OH, R_2 is $OCOCH_3$ and R_3 is $OCOCH_3$.



	R_1	R_2	R_3	IC_{50}
SL0101-1	OH	OAc	OAc	90 nM
SL0101-2	OAc	OH	OAc	580 nM
SL0101-3	OH	OH	OAc	190nM

FIGURE 1

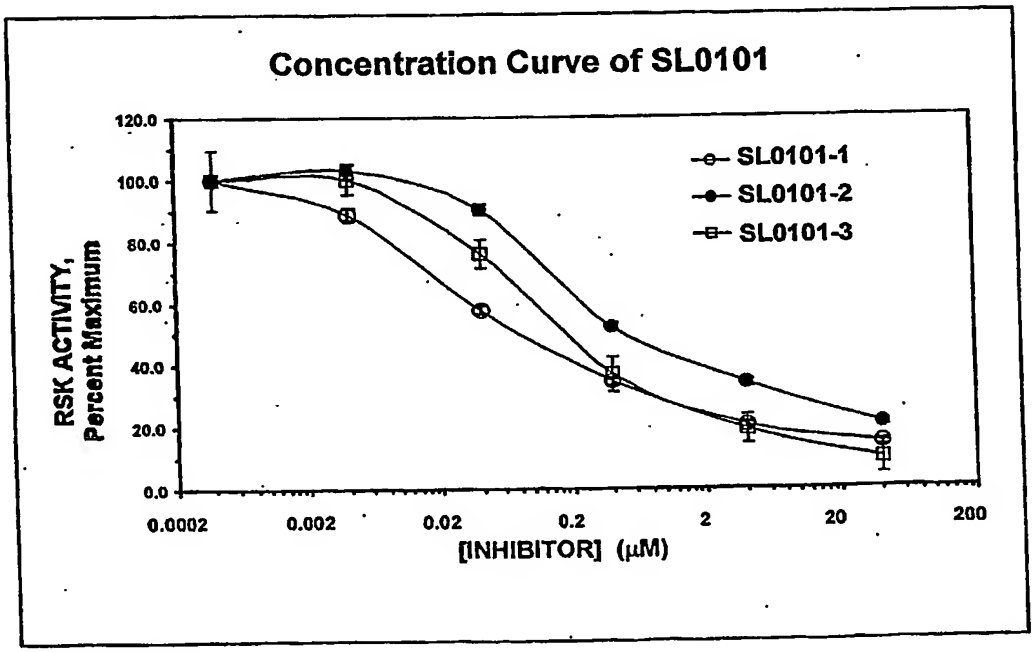


FIGURE 2

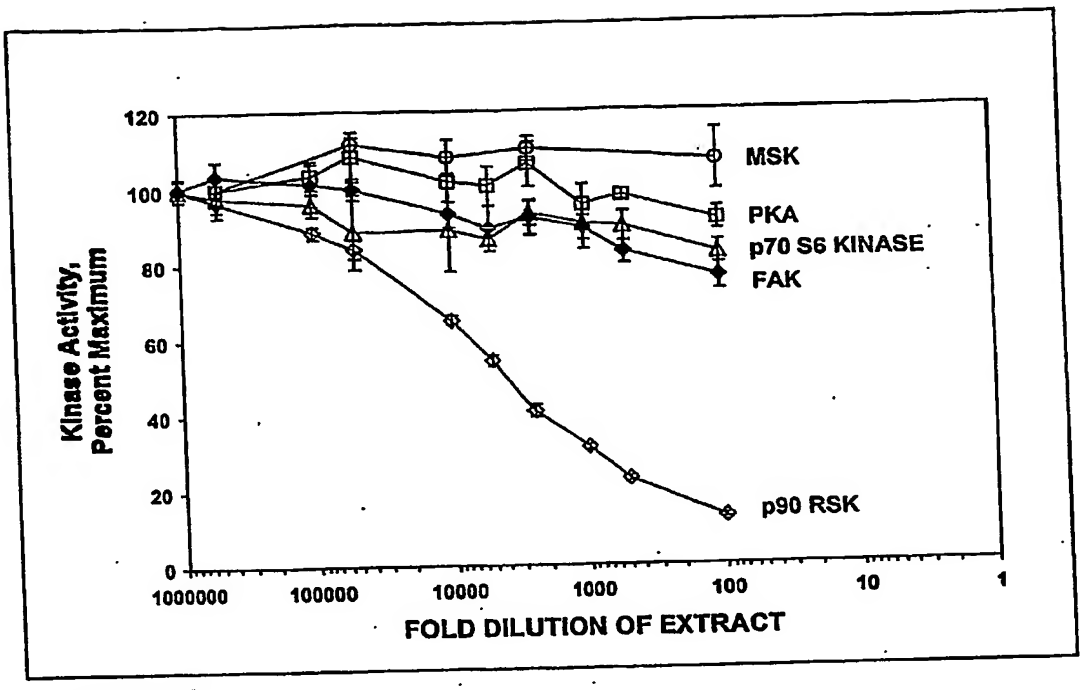


FIGURE 3

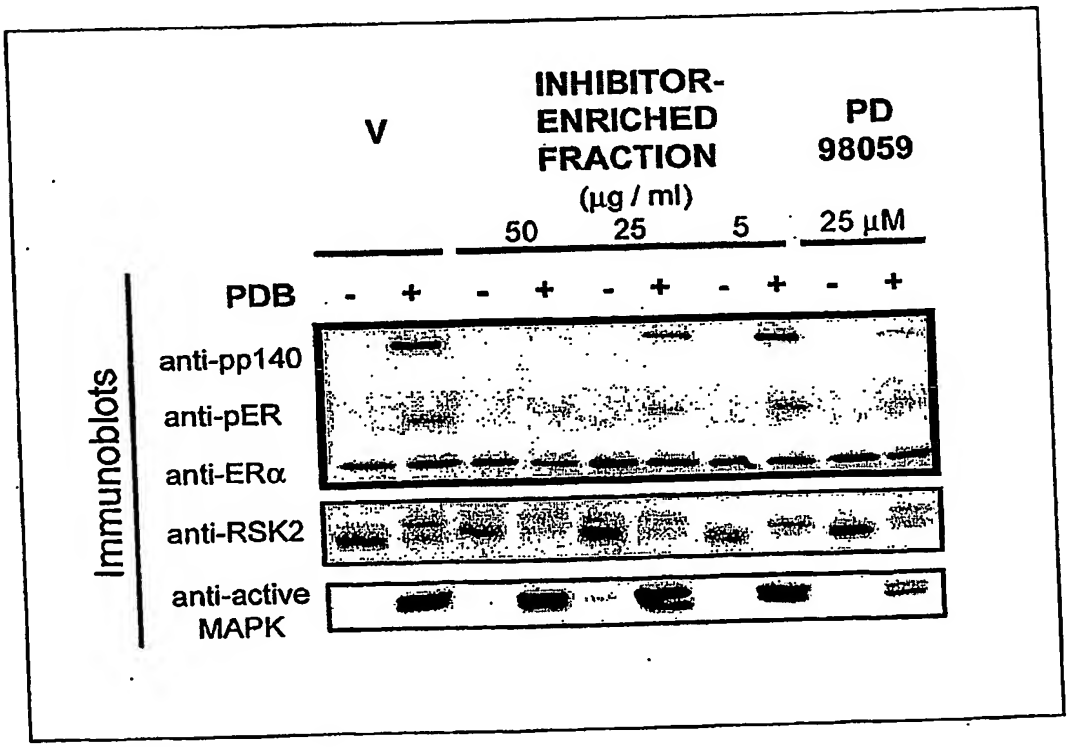


FIGURE 4

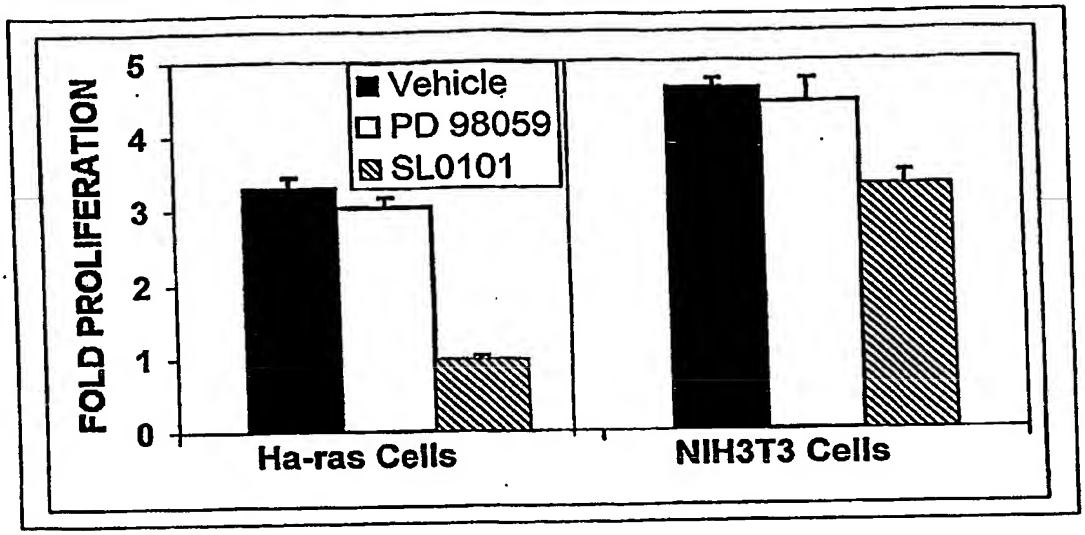
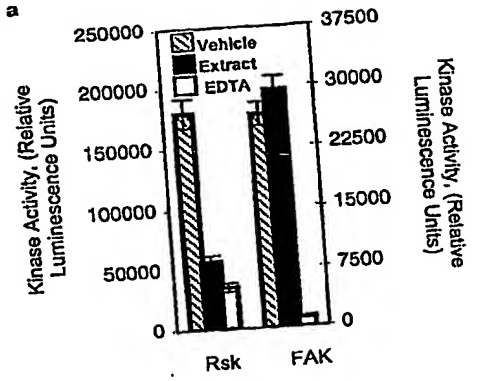
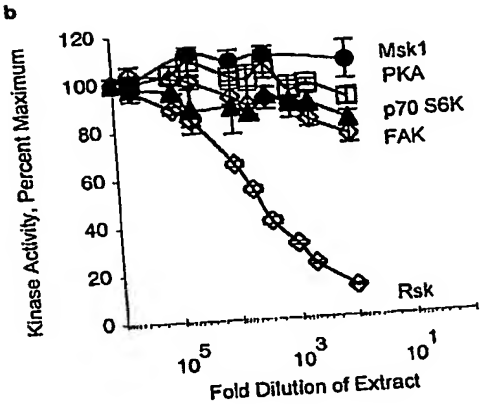
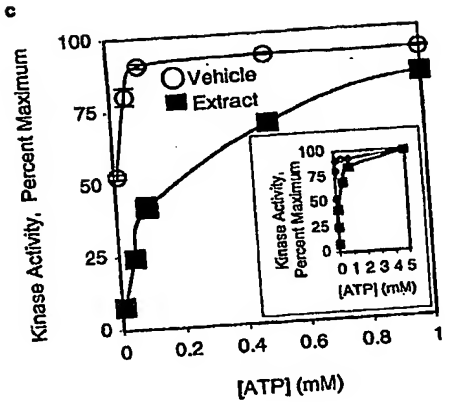

FOLD PROLIFERATION
5
4
3
2
1
0
Vehicle
PD 98059
SL0101
Ha-ras Cells
NIH3T3 Cells

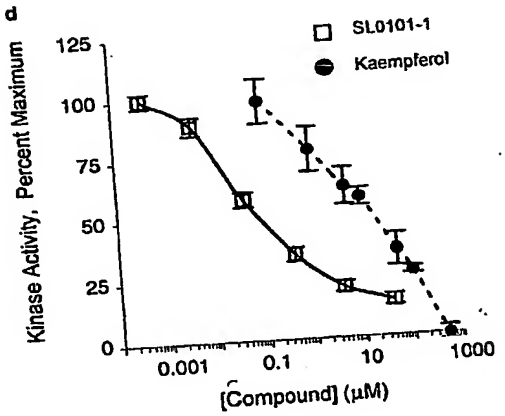
FIGURE 5

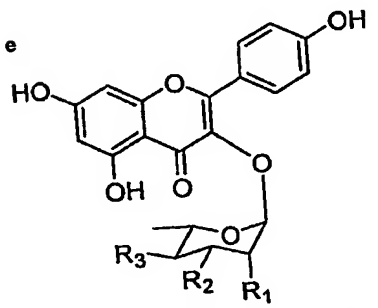
FIGURE 6











	R1	R2	R3	IC50
SL0101-1	OH	OAc	OAc	89 nM

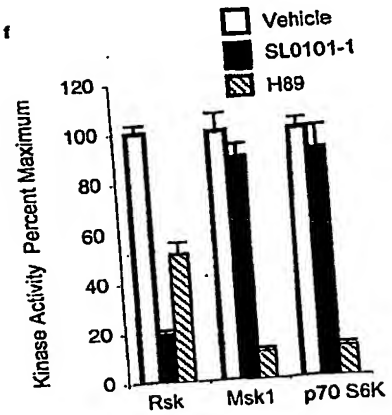
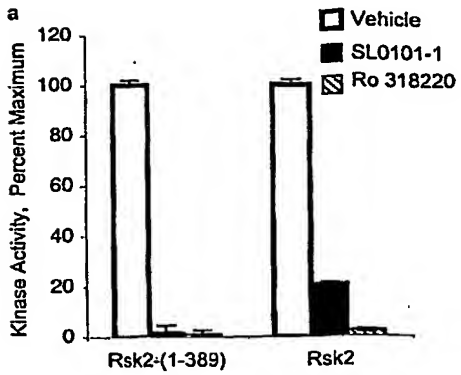
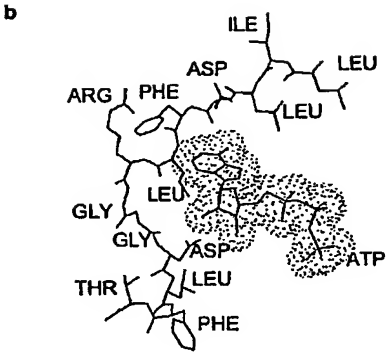


FIGURE 7


a
Vehicle
SL0101-1
Ro 318220
Kinase Activity, Percent Maximum
120
100
80
60
40
20
0
Rsk2-(1-389)
Rsk2


b
ILE
ASP
LEU
ARG
PHE
LEU
LEU
GLY
GLY
ASP
ATP
THR
LEU
PHE

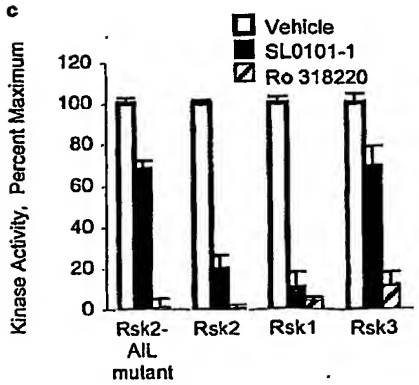

c
Vehicle
SL0101-1
Ro 318220
Kinase Activity, Percent Maximum
120
100
80
60
40
20
0
Rsk2-AIL mutant
Rsk2
Rsk1
Rsk3

FIGURE 8

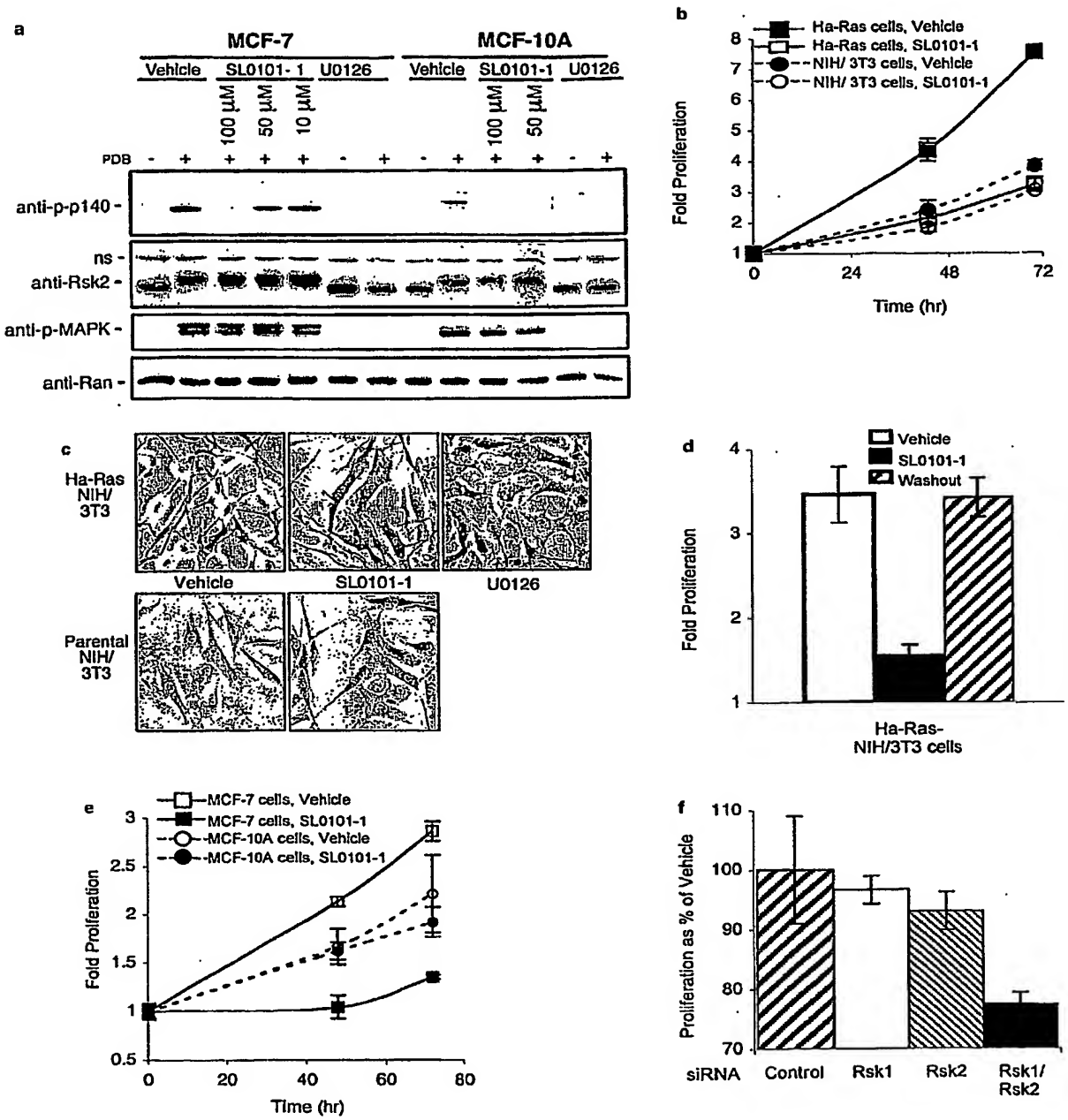

a
MCF-7
MCF-10A
Vehicle
SL0101- 1
U0126
Vehicle
SL0101-1
U0126
100 µM
50 µM
10 µM
100 µM
50 µM
PDB
anti-p-p140
ns
anti-Rsk2
anti-p-MAPK
anti-Ran
b
Ha-Ras cells, Vehicle
Ha-Ras cells, SL0101-1
NIH/ 3T3 cells, Vehicle
NIH/ 3T3 cells, SL0101-1
Fold Proliferation
Time (hr)
c
Ha-Ras NIH/ 3T3
Vehicle
SL0101-1
U0126
Parental NIH/ 3T3
d
Vehicle
SL0101-1
Washout
Fold Proliferation
Ha-Ras-NIH/3T3 cells
e
MCF-7 cells, Vehicle
MCF-7 cells, SL0101-1
MCF-10A cells, Vehicle
MCF-10A cells, SL0101-1
Fold Proliferation
Time (hr)
f
Proliferation as % of Vehicle
siRNA
Control
Rsk1
Rsk2
Rsk1/ Rsk2

FIGURE # 9

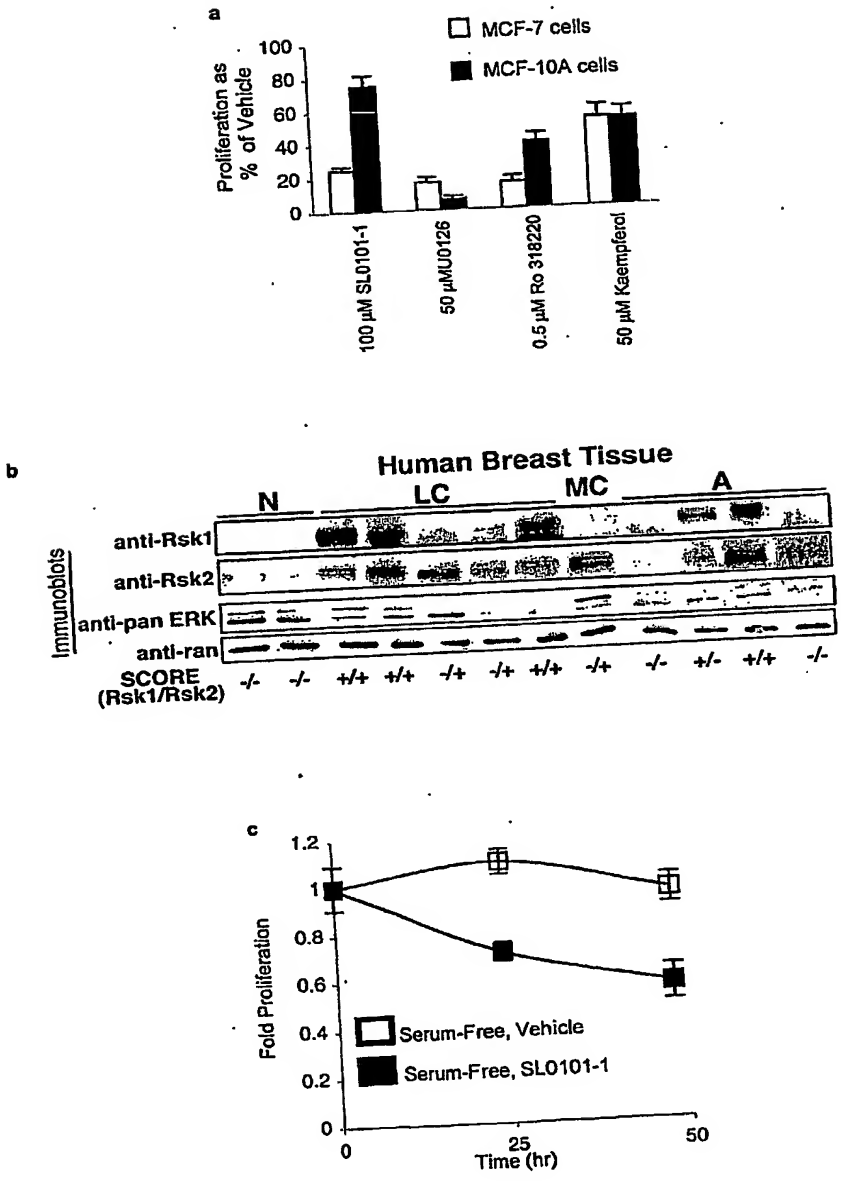

a
MCF-7 cells
MCF-10A cells
Proliferation as % of Vehicle
100
80
60
40
20
0
100 µM SL0101-1
50 µMU0126
0.5 µM Ro 318220
50 µM Kaempferol
b
Human Breast Tissue
N
LC
MC
A
anti-Rsk1
anti-Rsk2
anti-pan ERK
anti-ran
Immunoblots
SCORE (Rsk1/Rsk2)
-/- -/- +/+ +/+ -/+ -/+ +/+ -/+ -/- +/- +/+ -/-
c
Fold Proliferation
1.2
1
0.8
0.6
0.4
0.2
0
Serum-Free, Vehicle
Serum-Free, SL0101-1
0
25
50
Time (hr)

Fig 10.

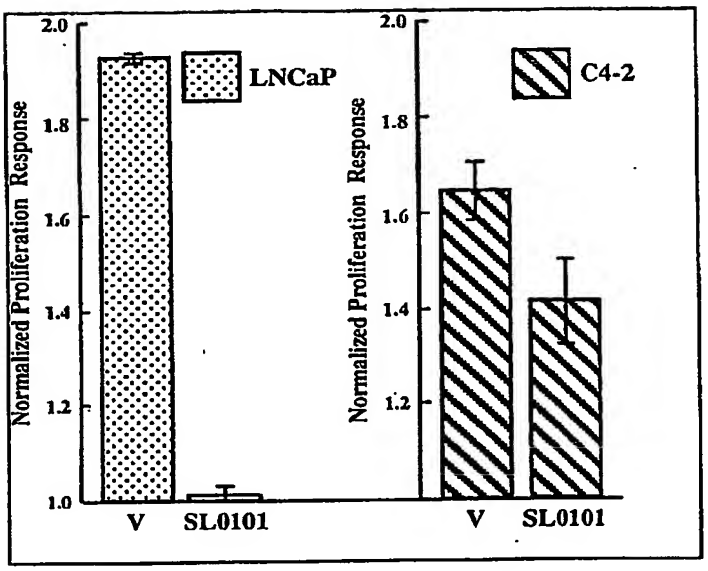

Normalized Proliferation Response
2.0
1.8
1.6
1.4
1.2
1.0
LNCaP
V
SL0101
C4-2
V
SL0101

Fig 11

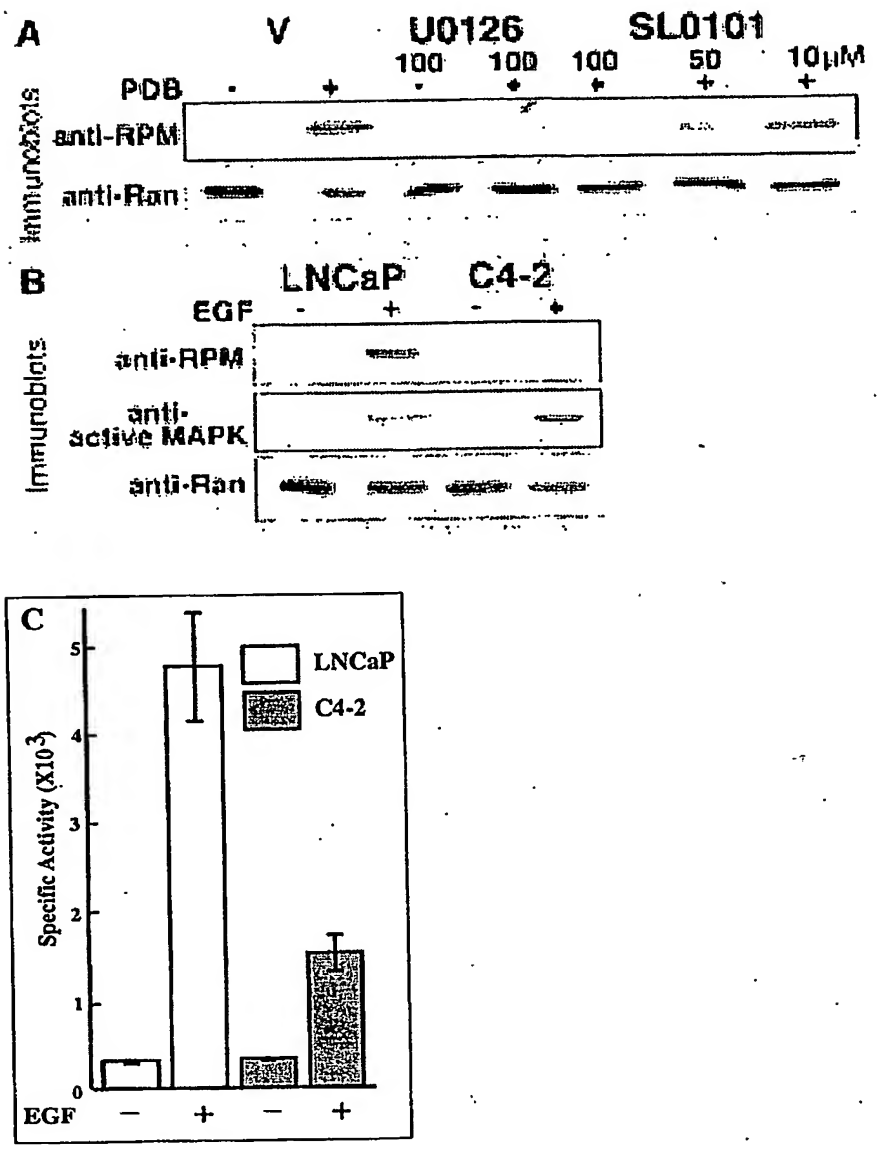
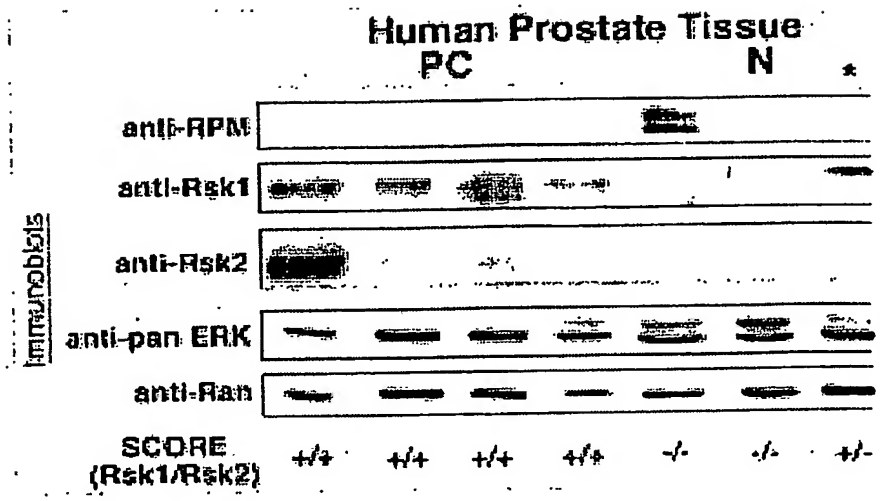

A
V
U0126
SL0101
100
100
100
50
10μM
PDB
anti-RPM
anti-Ran
Immunoblots
B
LNCaP
C4-2
EGF
anti-RPM
anti-active MAPK
anti-Ran
Immunoblots
C
LNCaP
C4-2
Specific Activity (X10-3)
0
1
2
3
4
5
EGF
−
+
−
+

Fig 12


Human Prostate Tissue
PC
N
anti-RPM
anti-Rsk1
anti-Rsk2
anti-pan ERK
anti-Ran
SCORE
(Rsk1/Rsk2)
+/+
+/+
+/+
+/+
-/-
-/-
+/-
Immunoblots

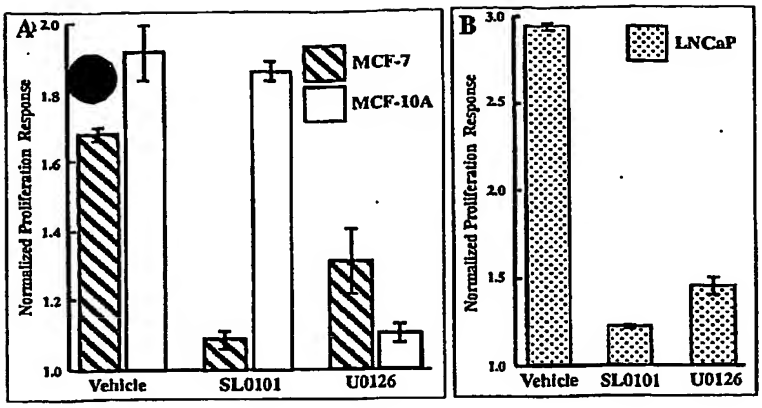
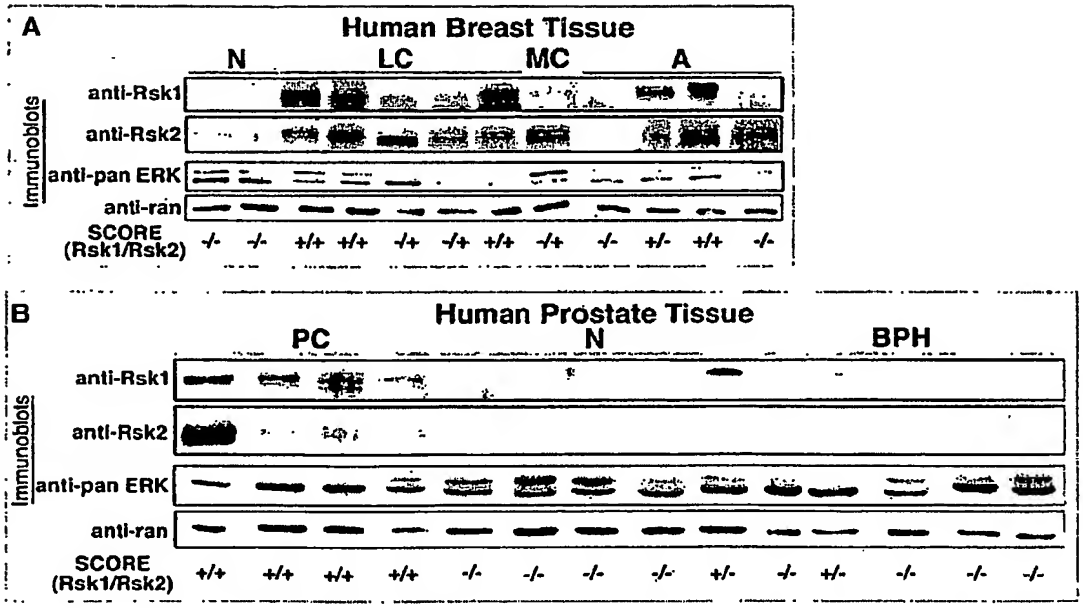


Fig.13 SL0101 inhibits the proliferation of cancer cells but not normal cells. (A) MCF-7 and MCF-10A cells and (B) LNCaP cells were treated with vehicle or 50 μM SL0101 or U0126 and a proliferation assay using CellTiter-Glo Luminescent Cell Viability Assay (Promega) was performed 44 hrs after treatment. The data are expressed relative to time 0.



described above.

Fig 14

CONFIDENTIAL

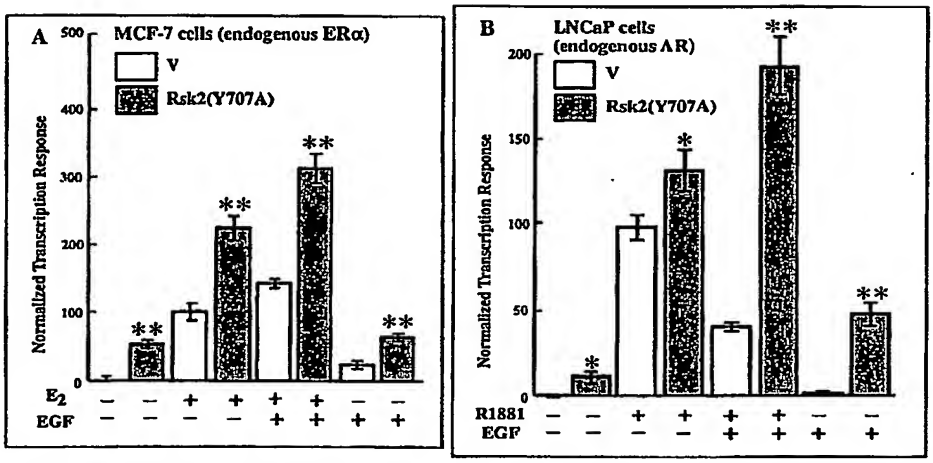


Fig.5 Rsk2 specifically activates ERα- and AR-mediated transcription. *(A) MCF-7 or (B) LNCaP cells were co-transfected with a luciferase reporter and β-galactosidase expression vectors. Additionally, the cells were transfected with either control vector (V) or a vector encoding constitutively active Rsk2 (Rsk2(Y707A)). The cells were treated with either vehicle (_), 10 nM estradiol or 5 nM R1881 and/or 100 ng/ml EGF. Luciferase and β-galactosidase activity were determined and the luciferase data were divided by the β-galactosidase activity to control for differences in transfection efficiency. The data were normalized so that, in the vector control, the response to vehicle addition was zero and the response to either estradiol or R18181 was 100. The values are ±SEM. *P<0.05 and **P<0.01 (Student's t-test) obtained by comparing the response obtained with the vector control with that obtained with Rsk2(Y707A).*